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18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number): bacterial spore; radiation resistance; heat resistance; spore germination; proteolysis.

19. ABSTRACT (Continue on reverse if necessary and identify by block number): Highlights of research findings in the past three years are: 1) using appropriate mutants lacking SASP we have shown that SASP-γ plays no role in spore heat or UV radiation resistance, and SASP-α, β and γ play no role in spore γ-radiation resistance; 2) we have shown that the transient high UV resistance attained during spore germination is dependent on the presence of SASP-α and β; 3) using immuno-electron microscopy we have shown that α/β-type SASP are associated with the forespore nucleoid, while SASP-γ is not; 4) we have achieved high level inducible expression of an α/β-type SASP in E. coli. This has allowed purification of large amounts of this SASP for physical studies of its interaction with DNA. Furthermore, synthesis of this SASP gives E. coli DNA some of the properties of DNA within spores!; 5) using a purified SASP we have converted the UV photochemistry of DNA in aqueous solution from thymine dimer production to spore photoproduct production; 6) physical studies of complexes formed between this purified α/β-type SASP and DNA have indicated that the DNA has been converted to the A conformation upon SASP binding; 7) we have cloned, sequenced and mapped the gene (termed gpr) coding for

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19. ABSTRACT

the SASP specific spore protease; 8) studies on the regulation of the gpr gene by in vitro and in vivo transcriptional mapping and with gpr-lacZ fusions have shown that gpr is transcribed first by $E\sigma^F$ and then by $E\sigma^G$; and 9) we have obtained high level expression of the B. megaterium protease zymogen in E. coli.

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Final Technical Report

The objectives and abstract of studies to be undertaken, as given in the initial grant application, were as follows:

Objectives

This project has two major objectives: 1) a detailed understanding of the role of various SASP in spore resistance to radiation and heat; and 2) an understanding at the molecular level of the processing (both its mechanism and regulation) and regulation of the SASP specific spore protease. Most of the work on spore resistance will be carried out with B. subtilis with some work also utilizing SASP genes or purified SASP of B. megaterium. While neither of these organisms are significant causes of food spoilage or food born diseases, much of the biochemistry and molecular genetics of SASPs and SASP genes is well understood in these organisms (as their SASP genes have been cloned and sequenced). In addition, molecular genetic manipulations of SASP genes in vitro, and subsequent introduction of altered genes into a chromosome in vivo is a simple process with B. subtilis, but is either not presently possible with most pathogenic species, or is extremely difficult. Consequently, because of the wealth of knowledge and techniques available for genetic manipulations in B. subtilis we will concentrate on this organism. Additionally, it seems very probable that the basic mechanism of spore resistance will be common among various gram positive spore formers. Consequently, it seems likely that any knowledge gained about spore resistance from studies with B. subtilis will be applicable to spore formers causing disease or food spoilage as well.

The work on the spore protease will begin using the gene for the B. megaterium spore protease, since this gene has been cloned. The B. megaterium gene will then be used as a probe to isolate the corresponding B. subtilis gene, and further studies on the spore protease will utilize B. subtilis exclusively. As noted above, the reasons for the use of B. subtilis are the wide variety of genetic and molecular genetic techniques and technology available for use with this organism. Again, it seems most likely that knowledge gained on the general details of the processing and regulation of the spore protease of B. subtilis, will also be applicable to other spore formers of more practical interest. Since at least one of the crucial steps in the processing of the spore protease takes place when spores are

becoming heat resistant, if we can duplicate this process in vitro it may provide detailed information on the environment within the spore during this period. This in turn may provide information on spore resistance as well.

Abstract

Using mutants of B. subtilis containing deletions in genes coding for two of the three major small, acid-soluble spore proteins (termed SASP-α and -β) we have recently shown that these SASPs play an essential role in the resistance of bacterial spores to ultraviolet irradiation, and at least an accessory role in spore heat resistance. We will extend these studies in a number of areas. 1) A deletion mutation in the gene for the third major B. subtilis SASP (termed SASP-γ) will be constructed in vitro using molecular genetic techniques. This mutation will be introduced into B. subtilis with loss of the wild type gene thus generating strains lacking only this SASP gene (γ-) or lacking all three genes which code for major SASP (α-β-γ-). 2) The phenotypes of these new strains will be investigated, especially with respect to their heat and UV radiation resistance, their ability to germinate and outgrow on rich and poor media and their appearance (in particular that of their DNA) in the electron microscope. We will also examine spores of the various SASP gene mutants for γ-radiation resistance. 3) If, as seems likely, the γ-mutation increases spore UV radiation sensitivity, we will determine the nature of the major DNA photoproducts formed by UV irradiation of various γ- strains. 4) Using plasmids carrying the cloned genes which code for SASP-α, β or γ we will re-introduce the wild type genes into appropriate SASP gene mutants and analyze the spores of such strains to obtain further information on unique or overlapping roles of individual SASP. 5) We will use the cloned SASP genes in appropriate expression vectors to produce large amounts of individual SASP. These SASP will then be used in experiments to duplicate in vitro the resistance of spore DNA to ultraviolet light. We will also attempt to crystallize individual SASP (in collaboration with Dr. Leo Herbette) and determine their crystal structure. 6) We will determine the nucleotide sequence of the gene coding for the SASP specific spore protease of B. megaterium, and clone, sequence and map the analogous gene from B. subtilis. We will then use the cloned B. subtilis gene to: a) introduce a deletion mutation in this gene into the chromosome and study the phenotype of the resulting strain, and

b) make large amounts of the enzyme zymogen for in vitro studies of the regulation of its processing.

During the three year period covered by this report we have made major progress on the objectives noted above, achieving a number of them. In addition, several new and promising lines of investigation have been initiated, and in some cases, completed. Highlights of this research are given below.

1) Cloning and analysis of the B. megaterium and B. subtilis gpr genes. The gene coding for the SASP-specific spore protease has been cloned and sequenced from B. megaterium and B. subtilis and also mapped on the chromosome. This work has allowed identification of one of the sites cleaved in the proteolytic activation of the zymogen form of this protease. Analysis of the regulation of this gene using lacZ fusions as well as in vitro and in vivo transcriptional mapping has shown that in vivo this gene is transcribed initially by $E\sigma^F$ and then by $E\sigma^G$. We have used the cloned genes in attempts to overexpress the zymogen forms of these enzymes to use as substrates to identify and characterize protease processing enzymes. To date this has been successful with the B. megaterium protease (3% of total protein in E. coli).

2) Role of α/β-type SASP in spore γ-radiation resistance. By use of strains lacking all three major SASP we found that $\alpha^-\beta^-\gamma^-$ spores had identical γ-radiation resistance to wt spores. This indicates that SASP play no role in γ-radiation resistance.

3) Role of α/β-type SASP in UV resistance during spore germination. Work in other labs has shown that during spore germination the spore goes through a period of extremely high UV resistance before its UV resistance falls to that of the growing cell. We found that spores lacking SASP-α and β do not show this transient elevated UV resistance during germination. This indicates that α/β-type SASP are required not only for dormant spore UV resistance, but also the elevated UV resistance during spore germination.

4) Localization of SASP within developing spores. Using immunoelectron microscopy we found that α/β-type SASP are associated exclusively with the forespore nucleoid. In contrast SASP-

γ is somewhat excluded from the nucleoid. This localization data is consistent with the known effects of α/β-type SASP (but not SASP-γ) on spore DNA properties in vivo.

5) Synthesis of SASP in E. coli. Using a plasmid with an inducible promoter we have achieved high level (1-2% of total protein) expression of various SASP in E. coli. This has allowed their efficient large scale purification (see below). In addition, while SASP-γ synthesis had essentially no effect in E. coli, synthesis of α/β-type SASP did. Upon synthesis of α/β-type SASP DNA replication stopped, plasmid DNA negative superhelicity increased 15-20%, and the cells nucleoid became extremely condensed and fibrillar. UV irradiation of these cells with high α/β-type SASP also gave significant levels of spore photoproduct (SP) and decreased levels of thymine dimers relative to uninduced cells. This data suggests that synthesis of α/β-type SASP has conferred significant spore like properties on the E. coli DNA.

6) Studies with purified SASP. Use of SASP purified as described above we have shown that these proteins bid non-specifically to DNA. The parameters affecting this binding (ionic strength, pH, divalent cation) have been determined as well as the kinetics of binding (slow) and stoichiometry. The latter is ~ one SASP/5 base pairs, similar to the level of SASP found in spores. Spectral studies of the structure of these SASP-DNA complexes, carried out in collaboration with Scott Mohr at Boston University, have shown that the DNA in these complexes is converted to the A-conformation. Studies of the UV photoproducts formed in these complexes have shown that only SP is formed - no TT. Thus we have duplicated spore DNA photochemistry in vitro in aqueous solution by binding an α/β-type SASP.

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